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Preliminary Characterisation of Immune Cell Populations in the Oral Mucosa of a Small Cohort of Healthy Dogs (*Canis lupus familiaris*)

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ABSTRACT

Pre-determined anatomical locations in the oral cavity were biopsied, and their histomorphology was characterised using haematoxylin and eosin staining (H&E). The most abundant cell type was of dendritic morphology. Lymphocyte foci were not evident in the palatoglossal folds or the gingiva. Immunohistochemical staining (IHC) for validated leukocyte markers followed, including CD3, CD20, CD79α, CD204, and Iba1. Consistent with H&E findings, CD204 immunoreactivity predominated amongst all niches. With the exception of the alveolar mucosa and palatoglossal folds, we also demonstrate a significant difference in the population of macrophages by region for only the Iba1 antigen ($p < 0.0001$). B lymphocytes were found, and a significant difference was noted in the sub-epithelium where CD20-positive cells outnumbered those labelled as CD79a positive ($p = 0.001$), suggesting the possibility that these cells are in an active state in health. A similar significant difference was found in the subepithelial tissue for myeloid cells, as there were more cells labelled as CD204 positive over Iba1, which, along with their distribution pattern, indicates a possible functional and morphological overlap between these cells. No significant difference was found in epithelial tissues for cells of either myeloid or lymphoid origins. The results from this study suggest different regions of the oral cavity exhibit variations in the distribution of immune cells, particularly macrophages and B lymphocytes. Though more studies would be needed to confirm these findings, these differences may have implications for the immune response and overall health of the oral mucosa.

1 | Introduction

The oral mucosa serves as a gateway to the body and offers first-line protection against environmental foes, including disease-causing microbes, chemicals, and airborne allergens. In addition to mechanical and chemical protective functions, the oral mucosa offers cell-mediated defence. The structural organisation of the oral immune cells in health, and specifically, the leukocytic cell populations in the oral mucosa of healthy dogs, has not been systematically studied, despite leukocytes' important role in host protection and disease. Therefore, there is clinical and research relevance in understanding the types and numbers of immune cells present in the various oral compartments of healthy dogs to better understand local events in the diseased tissues. Benefit from this knowledge has previously been provided after studying the immune cells present in the oral mucosa of cats (Arzi et al. 2011). Specifically, studies on feline gingivostomatitis, feline resorptive lesions

and squamous cell carcinoma all relied on these fundamental data (Arzi et al. 2010, 2016, 2017, 2020; Sparger et al. 2018).

It's important to note that immune cell populations vary not only across different regions of the body but also within close distances within the same region. For example, in the dog's intestinal tract, B cells and plasma cells predominate in peri-crypt regions, while lamina propria T cells and subsets of immune cells are more abundant in the tip of the villus (German, Hall, and Day 1999). Characterisation of immune cell types of three sites within the oral cavity (buccal, sublingual, and lingual tonsil) in non-human primates shows T-cells predominating in sublingual tissue and the tonsil but are less prevalent in buccal samples, while B-cells accounted for a higher percentage of CD45+ leukocytes in the tonsils (Hernandez et al. 2022). Similar variability in leukocytic cell distribution was reported by Arzi et al. (2010) in the oral cavity of specific pathogen-free cats as well as in relation to health versus disease (Arzi et al. 2011). Based on the knowledge of possible differences in disease susceptibility between dogs and cats (Day 2016) and the lack of references on oral immune cell distribution in canine species, we designed this study.

Immunology studies, on the other hand, are somewhat limited in canine species due to a deficiency in specific antibodies required for immunophenotyping. However, using currently available and validated markers, we can bridge some knowledge gaps. CD3 (cluster of differentiation 3) is a protein complex and T cell co-receptor that is involved in activating both the cytotoxic T cell and T helper cells. Immunoreactivity for this marker confirms T cell origin (Keller et al. 2013; Noland, Keller, and Kiupel 2018; Sueiro, Alessi, and Vassallo 2004). CD20 (cluster differentiation 20) is expressed on the surface of all B-cells, beginning at the pro-B phase and progressively increasing in concentration until maturity. Immunoreactivity for this marker confirms B cell origin (Anderson et al. 2020). CD79a (cluster differentiation 79a) is the signal transduction portion of the B-cell receptor that spans the membrane and intra-cytoplasmic regions of the cell. This receptor is expressed in all immature and mature B-cells and in the majority of B-cell neoplasms (van Noesel et al. 1991; Mason et al. 1995). This is a highly reliable marker for B cells in immunohistochemical staining (IHC), whereas CD20 can also be found on some T cells and myeloid blast cells (Anderson et al. 2017; Mason et al. 1995). CD204 (macrophage scavenger receptor 1) is a pattern recognition receptor that is capable of binding to a large variety of ligands and is present in both dendritic cells as well as M2 polarised macrophages (Anderson et al. 2020; Gudgeon, Marín-Rubio, and Trost 2022). Iba1 (allograft inflammatory factor 1) is a macrophage-specific calcium-binding protein, and immunoreactivity for this marker indicates macrophage activation (Pierezan et al. 2014). These markers were selected because they are widely available and validated for use on formalin-fixed tissues.

This study aims to fill a knowledge gap by providing a comprehensive topographical and quantitative analysis of the distribution and histological frequencies of immune cells in the healthy oral mucosa of dogs. Our hypothesis is that niches with different functional capacities will have distinct cell populations, that myeloid cells will be most abundant and that lymphoid foci will be found in some of these niches. The data obtained will enable comparisons to be made in health and disease.

2 | Materials and Methods

2.1 | Study Animals

Cadaver heads from eight dogs euthanised for reasons unrelated to this study were evaluated for inclusion in this study. All of the studies were conducted according to approved protocols set up by the Animal Use and Care Administrative Advisory Committee, University of California, Davis. Only four of these specimens met inclusion criteria. A board-certified dentist and oral surgeon (MSR) qualified the specimens to assure they were free of orodental disease such as attachment loss, osteomyelitis or necrosis, oral neoplasia, or tooth resorption via dental charting and imaging of dental and maxillofacial structures utilising cone beam computed tomography (CBCT). Specimens were excluded if any of these diseases were noted or if a history of systemic disease that could affect the oral cavity or immune system was identified. Two heads were stored frozen (20°C) prior to sampling, and two heads were sampled immediately after euthanasia.

2.2 | Oral Compartment Tissue Samples

Samples were obtained utilising Metzenbaum surgical scissors, Adson Tissue forceps, a P24G periosteal elevator and an 8 mm biopsy punch. Frozen heads were defrosted at room temperature prior to sampling. Samples were obtained bilaterally, once from the right side of the oral cavity and once from the left, for 11 pre-determined locations (i.e., niches) in the oral cavity, including the mucosa and submucosa (i.e., compartments) (Figure 1). Harvested samples of less or equal to three millimetres were placed in biopsy cassettes submerged in 10% neutral buffered formalin solution to be fixed en-bloc. Collected samples underwent H&E staining as previously reported (Arzi et al. 2011). A board-certified pathologist (NVA) analysed these slides for neutrophil, mast cell and eosinophil counts, as well as for the presence of lymphocyte foci. The morphology of the tissues was also analysed. Left and right sides were compared per site, and the side with the highest cellularity was chosen for the subsequent immunohistochemical (IHC) analysis.

2.3 | Immunohistochemistry

Immunohistochemical analysis was done for the following antigens: CD3, CD20, CD79α, CD204 and Iba1, as previously reported (Table 1) (Anderson et al. 2017, 2020; Keller et al. 2013; Kim et al. 2014; Noland, Keller, and Kiupel 2018; Pierezan et al. 2014; Sueiro, Alessi, and Vassallo 2004). Samples were placed on slides, fixed for five minutes in acetone, followed by being air-dried and immersed within 0.3% hydrogen peroxide and 0.1% sodium azide for a period of ten minutes in order to prevent endogenous peroxidase activity. Then, for a period of twenty minutes, non-specific antibody interactions were blocked through immersion in 10% normal horse serum. In a mixture of phosphate-buffered saline with 10% inactivated horse serum, all of the antibodies were applied as a 1:10 diluted tissue culture. These slides were placed in incubation along with anti-canine primary antibodies for one hour. Next, each section received secondary biotinylated horse anti-mouse IgG, which was followed by being stained with the biotin-streptavidine-horseradish-persoxidase method, while the

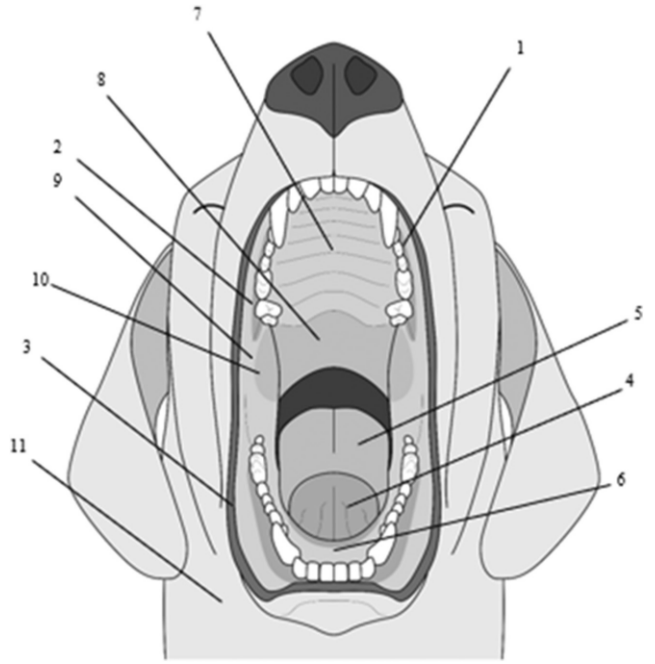

FIGURE 1 | Diagram of the topographic locations (i.e., niches) biopsied: 1. Attached gingiva, 2. Alveolar mucosa, 3. Buccal mucosa, 4. Ventral surface of the tongue, 5. Dorsal surface of the tongue, 6. Floor of the mouth, 7. Hard palate, 8. Soft palate, 9. Palatoglossal folds, 10. Palatine tonsils, 11. Mandibular lymph nodes. Bilateral samples were obtained from each location. Mandibular lymph nodes and palatine tonsils served as controls.

chromogen that was used was three-amino-9-ethylcarbazole. The counterstain was performed for each section using Mayer's haematoxylin, with both positive and negative control tissues being made for each experiment. Canine mandibular lymph nodes and tonsils containing multiple leukocyte lineages acted as positive and negative controls. Negative controls were made by omitting the primary antibody and instead using an isotype control antibody. Each slide created had a sample placed along with a control to allow for direct comparison.

2.4 | Quantification of Cell Populations

Different cell populations were quantified by two investigators (MB and MSR) and confirmed by a board-certified veterinary pathologist (NV) by assessing the H&E- and IHC-stained slides. Specifically, four high-resolution photomicrographs were obtained using an Olympus microscope (Olympus Optical Co., LTD, model: BX40F4) with a colour camera attachment (Olympus DP72) and CellSense/Olympus software. Each photomicrograph represents a total area of ~160,000 μm^2 (0.16 mm^2). A manual count of immunoreactive cells for each of the five antigens, including CD3, CD20, CD79α, CD204 and Iba1, was then performed with Fiji/ImageJ (ImageJ Public Licence, imagj.nih.gov, Version 1.51 23). Each positively labelled cell was identified and quantified as the average number of these cells per μm^2 of epithelium scrutinised, as well as per μm^2 of lamina propria/submucosa scrutinised. Counts were then averaged for the number of cells stained by each of the five antigens at each of the nine sites tissues were harvested from. We relied on cytoplasmic

TABLE 1 | Antigens with their associated sources, validation studies, suppliers, catalogue number, concentration, dilution factor and specificity.

Antigen	Source	Validation studies	Supplier	Catalogue#; clone	Concentration	Horse serum volume (μL)	Antigen volume (μL)	Dilution factor	Specificity
CD3	Rat	(Keller et al. 2013; Sueiro, Alessi, and Vassallo 2004; Noland, Keller, and Kiupel 2018)	LABL	CA17.2A12 and CD3-12	5–10 μg/mL	300	3	1:100	T cell
CD20	Rabbit	(Anderson et al. 2020)	Thermo-Fisher	12673297; RB-9013	200 μg/mL	300	1	1:300	B cell
CD79α	Mouse	(Anderson et al. 2017)	BioRad	MCA2538H; HM57	0.1 mg/mL	300	3	1: 100	B cell
CD204	Mouse	(Anderson et al. 2020)	TransGenic	KT022; SRA-E5	5 μg/mL	300	1.5	1:200	Macrophages
Iba1	Rat	(Pierezan et al. 2014)	Wako	19-19741	0.5–0.7 mg/mL	300	0.6	1:500	Macrophages/ dendritic cells

immunoreactivity to count a cell as positive. The 204 antigen is present on macrophage cells, and their morphology, especially in resident tissue macrophages, can be fibroblast-like with multiple cytoplasmic projections. Even if immunoreactive processes were observed, only cells with obvious nuclei and immunoreactive cytoplasmic staining were counted.

2.5 | Statistical Analysis

In summary, samples were collected from 11 specific locations on both sides of the oral cavity, resulting in 22 H&E slides per head, with the side showing higher cellularity at each site selected for further immunohistochemical (IHC) analysis, thus evaluating 22 areas per patient when considering both the epithelium and lamina propria separately. This dataset was analysed by a statistician (AB) through repeated measures ANOVA with the factors of tissue sample region, specific aspect of the tissue, be it epithelial or submucosa, and cell type based on the five antigens. As the goal is cell count, we considered a generalised linear mixed effect model (GLMM) with random intercept for each dog using the negative binomial distribution as the response distribution due to the presence of overdispersion. This analysis was done in the program, RStudio, using the glmmTMB R package (Brooks et al. 2017; R Core Team 2023).

3 | Results

Of the four specimens that met inclusion in the study, one was a Labrador retriever, one was a Labrador retriever mix, one was a Siberian huskie, and one was a Rottweiler mix. Age was estimated to be between 11 months and 5 years old based on root canal width and apical closure, but gender and neuter status were unknown.

Investigation of the H&E-stained sections showed normal histomorphology features typical for each oral niche and compartment (Figure 2). Lymphoid foci were not found in gingiva or palatoglossal folds. Lastly, mast cells were not observed in any H&E sections; hence, no special stains for detection of these cells were applied. The attached gingiva is overlain by a keratinised squamous epithelium. The thick epithelial layer has prominent intradermal projections (rete pegs). The subepithelial stroma comprises tightly packed collagenous bundles and small calibre blood vessels. Alveolar mucosa is also overlain by a keratinised squamous epithelium, but it is less thick than the epithelium of the attached gingiva and does not have prominent rete pegs. The buccal mucosa is covered by non-keratinised squamous epithelium, and the submucosa comprises more loosely arranged collagen bundles. In some sections, it is not uncommon to observe salivary glands, acini and ducts, as well as skeletal muscle bundles. The tongue's ventral surface comprises

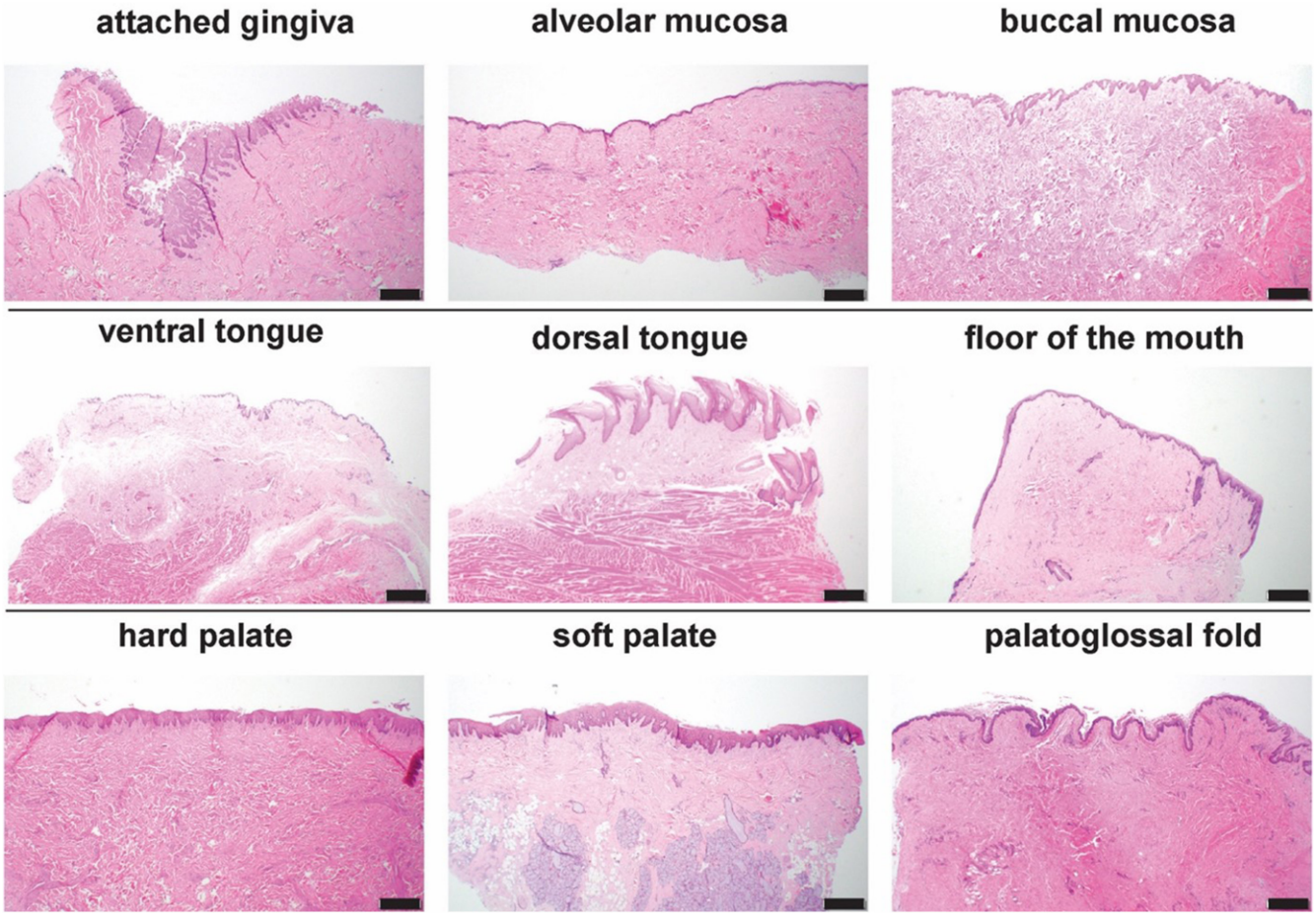

FIGURE 2 | Haematoxylin and eosin-stained section representing all nine niches. Control niches are not included (palatine tonsils and mandibular lymph nodes). Magnification 200× Bar = 500 μm.

a thin layer of non-keratinised squamous epithelium covering loose fibrous connective tissue subtended by a skeletal muscle. On the contrary, the squamous epithelium of the dorsal surface of the tongue is thick, heavily keratinised and forms saw-like structures. The submucosa is comprised of loose fibrous connective tissue and contains larger calibre arterioles. Submucosa then transitions into the skeletal muscle, where striated muscle bundles are arranged in orthogonally intercepting plains. The floor of the mouth has a thin epithelial lining comprised of non-keratinised squamous epithelium subtended by moderately vascularised fibrous connective tissue with loosely packed collagen bundles. The hard palate tissue comprises a thick layer of keratinised squamous epithelium subtended by a minimally vascularised fibrous connective tissue where collagen bundles are tightly packed. The epithelial layer has prominent rete pegs that extend and anchor itself in the subjacent subepithelial stroma. The soft palate has an epithelial lining identical to the hard palate. However, the subepithelial stroma is much looser and contains islands of salivary glands interspersed by adipose tissue. A keratinised squamous epithelium is covering the palatoglossal folds. The subepithelial stroma is represented by densely packed collagen fibres intermingled with small calibre blood vessels.

3.1 | Immunohistochemical Staining Reveals a Predominance of CD204 Cells and a Difference in Frequency of Iba1 Cells in Different Niches

Each of the nine oral anatomical niches or locations was examined for the types and quantities of certain immune cells found in each. Figure 3 presents the quantity of positively labelled cells per μm^2 for each of the five antigens evaluated (CD3, CD20, CD79α, CD204 and Iba1) for each of the niches evaluated. The epithelium

Epithelium (41872μm²)	Attached Gingiva (AG)	Alveolar Mucosa (AM)	Buccal Mucosa (BM)	Ventral Tongue (VT)	Dorsal Tongue (DT)	Floor of Mouth (FM)	Hard Palate (HP)	Soft Palate (SP)	Palatoglossal Fold (PGF)
CD3	9	5.75	8.25	3.75	2.67	3.75	4.5	3.5	3.33
CD20	0	0	0	0	0	0	0	0	0
CD79a	0	0	0	0	0	0	0	0	0
CD204	2	3	4.75	1.5	7.33	3.75	1.75	1.75	2.67
Iba1	4.25	7.25	3	1.5	3	10.25	1.25	2	2.33
Lamina Propria (115948μm²)	Attached Gingiva (AG)	Alveolar Mucosa (AM)	Buccal Mucosa (BM)	Ventral Tongue (VT)	Dorsal Tongue (DT)	Floor of Mouth (FM)	Hard Palate (HP)	Soft Palate (SP)	Palatoglossal Fold (PGF)
CD3	6.75	6.75	2.25	2.75	3.25	7.5	8.25	3.25	13
CD20	2.25	1.25	0.5	0.25	1.5	1	0.75	2.5	19
CD79a	1	0.33	0	0.33	1.67	0.33	0	0	0.5
CD204	29	50.5	38.25	56	51	70.5	33.5	71	64.33
Iba1	7.5	11.5	1.5	5.25	3.25	30	6.5	10.5	20

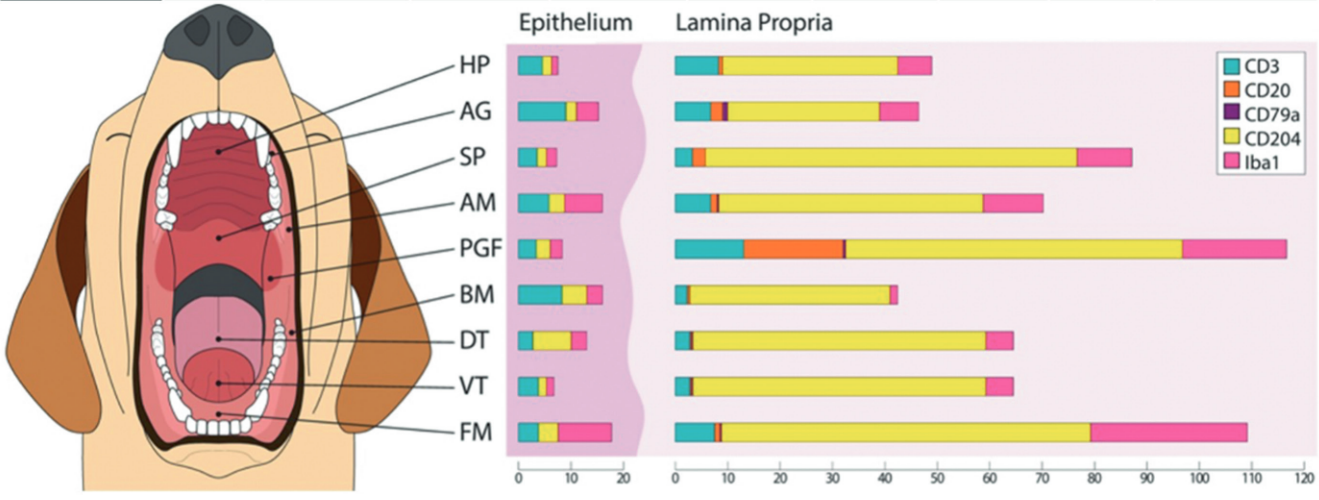

FIGURE 3 | Average cell density (cells/per area in μm^2) by immunoreactivity and niche comparing the epithelial and submucosal compartments. Average area of epithelium (EAV) evaluated was 41,872 μm^2. Average area of lamina propia evaluated was 115,948 μm^2.

and subepithelium were evaluated as separate compartments. Within the epithelial compartment of the attached gingiva, the most frequently encountered cells were CD3 positive, followed by CD204 and Iba1, with no cells labelled with CD20 or CD79α. In the lamina propria, CD204-positive cells were most frequent, followed by Iba1 and CD3, with scant CD20 and CD79α. In the epithelial compartment of the alveolar mucosa, Iba1-positive cells were most common, followed by CD3 and CD204, again with no cells labelled with CD20 or CD79α. The lamina propria showed CD204 as most frequent, followed by Iba1 and CD3, with scant CD20 and CD79α. In the buccal mucosa, CD3 cells were most common in the epithelial compartment, followed by CD204 and Iba1, with no CD20 or CD79α labelling. The lamina propria had CD204 as most frequent, followed by CD3, CD20 and Iba1, with no CD79α. For the ventral tongue, CD3 was most common in the epithelial compartment, followed by CD204 and Iba1, with no CD20 or CD79α. The lamina propria showed CD204 most frequently, followed by Iba1 and CD3, with scant CD20 and CD79α. In the dorsal tongue, CD204 cells were most common in the epithelial compartment, followed by CD3 and Iba1, with no CD20 or CD79α. The lamina propria had CD204 most frequently, followed by Iba1 and CD3, with scant CD20 and CD79α. In the floor of the mouth, Iba1 was most common in the epithelial compartment, followed by CD3 and CD204, with no CD20 or CD79α. The lamina propria showed CD204 most frequently, followed by Iba1, with scant CD3, CD20 and CD79α. For the hard palate, CD3 was most common in the epithelial compartment, followed by CD204 and Iba1, with no CD20 or CD79α. The lamina propria had CD204 most frequently, followed by CD3 and Iba1, with scant CD20 and no CD79α. In the soft palate, CD3 was most common in the epithelial compartment, followed by CD204 and Iba1, with no CD20 or CD79α. The lamina propria showed CD204 most frequently, followed by Iba1, with scant CD3 and CD20, and no CD79α. For the palatoglossal fold, CD3 was most common in the epithelial compartment, followed by CD204 and Iba1, with no CD20 or CD79α. The lamina propria had CD204 most frequently, followed by Iba1, CD3 and CD20, with scant CD79α. Palatine tonsil and mandibular lymph nodes served as positive tissue controls, verifying labelling for CD3, CD20, CD79α, CD204 and Iba1. The palatine tonsillar epithelium was the only compartment where CD20-positive cells were detected. Iba1-labelled cells were more frequent in intraepithelial cells than CD204, whereas subepithelial immunopositivity for CD204 was higher than Iba1 in control tissues, similar to the areas examined.

3.1.1 | Immunoreactivity in the Epithelium as Compared to the Lamina Propria

When evaluating all markers, CD3-positive cells predominated in the epithelial compartment, followed by Iba1 and CD204-positive cells for all niches. CD204-positive cells predominated in the lamina propria, followed by Iba1, CD3 and CD20-positive cells for all niches. Considering lymphocyte populations alone,

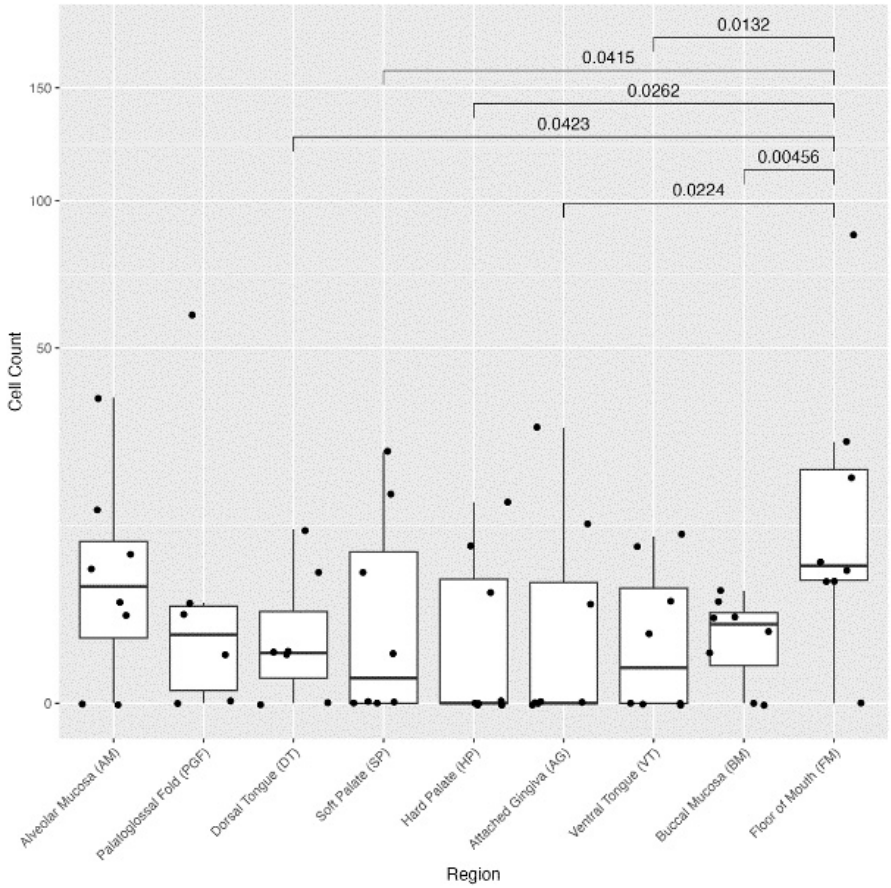

FIGURE 4 | Cell count for Iba1 immunoreactivity in the epithelium and lamina propia for each of the nine niches evaluated. The line in the centre of the box denotes the median, while the error bar (i.e., whiskers) represents the minimum and maximum value that is not an 'outlier' (i.e., not more than 1.5 × interquartile range away from the 25th percentile and the 75th percentile set as the defaults in R).

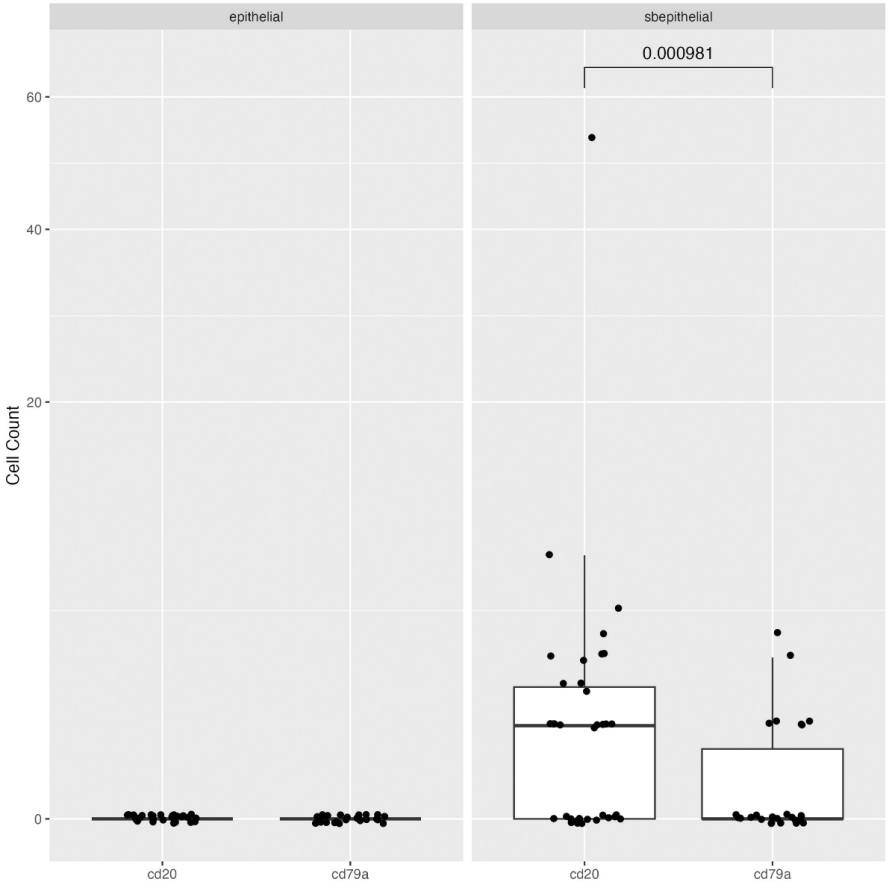

FIGURE 5 | Cell count of CD20 and CD79α lymphocytes in the epithelial and subepithelial compartments. The line in the centre of the box denotes the median, while the error bar (i.e., whiskers) represents the minimum and maximum value that is not an 'outlier' (i.e., not more than 1.5 × interquartile range away from the 25th percentile and the 75th percentile set as the defaults in R).

these varied in the epithelium and lamina propria Figure 3. The epithelium showed exclusively CD3 immunoreactivity, whereas the lamina propria showed immunoreactivity to both CD3 and CD20, with a predominance of CD3 over CD20-positive cells in all niches but the palatoglossal folds. These differences were not statistically significant.

3.1.2 | Differentiation by Niche and Compartment

Only Iba1 was found to have a significant difference ($p < 0.0001$) by niche amongst the five antigens examined (Figure 4). A significant difference ($p = 0.001$) was also found between lymphocytes in the submucosal tissue as there was a larger number of CD20 than CD79α, yet there was no significant difference found between lymphocytes in the epithelial tissue (Figures 5 and 6). Evaluation of myeloid cells showed a significant difference ($p < 1e\text{-}24$) between CD204 and Iba1 in the submucosal tissue with a larger number of CD204 labelling as compared to Iba1, yet there was no significant difference between the two in the epithelial tissue (Figures 7 and 8).

4 | Discussion

This study aimed to provide a comprehensive topographical and quantitative analysis of the distribution and histological

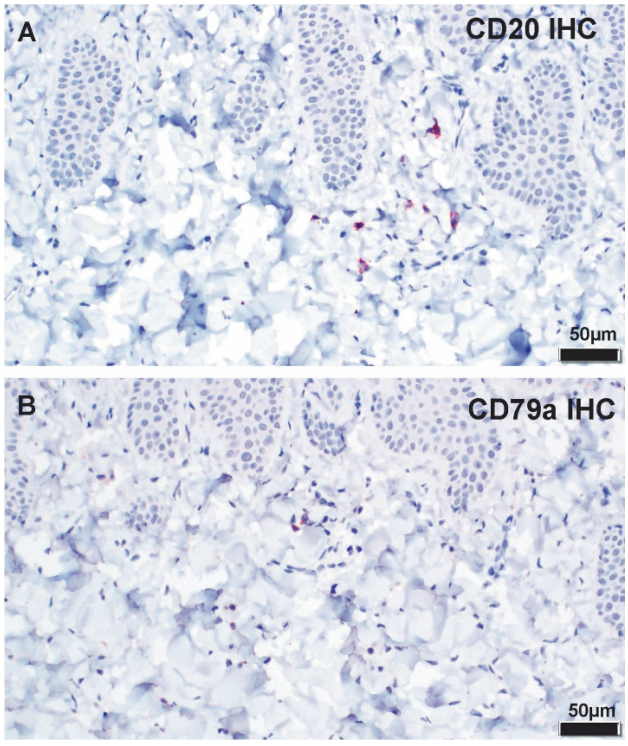

FIGURE 6 | Attached gingiva (200× magnification; bar = 50 μm). (A) IHC labelling for CD20. (B) IHC immunolabelling for CD79α antigen (red). Note all immunoreactive cells are located in the submucosa.

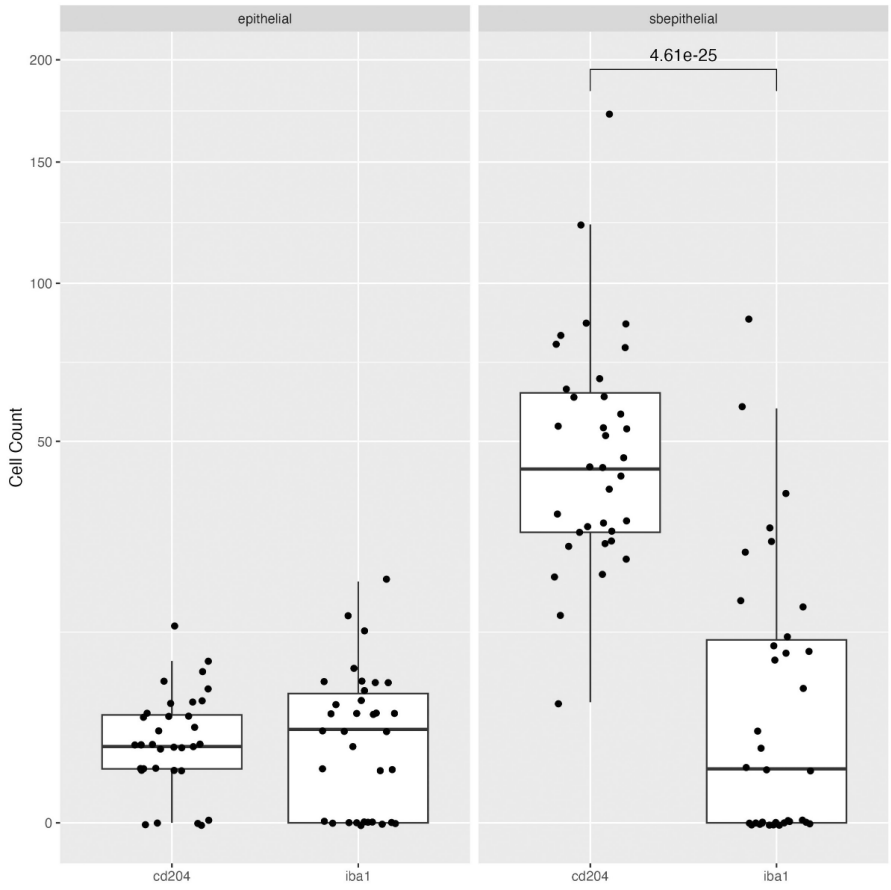

FIGURE 7 | Cell counts of leukocytes immunoreactive for CD204 and Iba1 in the epithelial and subepithelial locations. The line in the centre of the box denotes the median, while the error bar (i.e., whiskers) represents the minimum and maximum value that is not an 'outlier' (i.e., not more than 1.5 × interquartile range away from the 25th percentile and the 75th percentile set as the defaults in R).

frequencies of different immune cells in the healthy oral mucosa of dogs. The key finding of this study is the variation Iba1+ cells (i.e., macrophages) exhibited across most oral niches. When analysing the data by compartments, the submucosal tissues exhibited a notable variation between CD204+ and Iba1+ cells, with the former surpassing the latter in number, while no difference in cell counts immunoreactive for these markers was detected in the epithelial compartment. Furthermore, T cells (CD3-positive) were detected in epithelial and subepithelial compartments, while B cells (CD20-positive and CD79a-positive) were detected almost exclusively within subepithelium. Additionally, CD20+ B cells outnumbered CD79α-positive B cells in the submucosal tissue. These observations are pivotal for our goal of unravelling the immunological patterns within the oral cavity of healthy dogs and our understanding of oral immunobiology in health and disease.

The predominant morphology for immune cells presenting in the oral cavity of dogs was dendritic. Dendritic cell morphology can be exhibited by dendritic cells or macrophages that are known for their role in antigen presentation to T cells (Steinman and Cohn 1973, 1974; Steinman, Lustig, and Cohn 1974). This is consistent with the finding that the most widely present morphology of immune cells in cats is dendritic cell morphology (Arzi et al. 2011). In humans, it has also been found that dendritic cells localise at the border zones to the environment, including in the oral cavity (Novak et al. 2010). The predominant immunoreactivity was for CD204. CD204, also called scavenger receptor A, is a phagocytic pattern-recognition receptor (PRR) expressed primarily on macrophages and dendritic cells (DCs). Dendritic cells in normal tissues do not express CD163 and CD204, although they are able to upregulate these receptors in diseased tissues (Yi et al. 2009). Macrophages, on the other hand, express an array of scavenger receptors, including CD163 and CD204. Macrophages are typically located in the lamina propria, as observed in our study. Evidence suggests plasticity to overlap in morphology, function and surface markers between macrophages and dendritic cells in tissues, including the oral cavity (Doebel, Voisin, and Nagao 2017; Gottschalk and Kurts 2015; Hume 2006; Metcalfe et al. 2022). Oral commensals generally elicit an M2-like phenotype, while oral pathogens elicit a more M1-like phenotype (Huang, Alimova, and Ebersole 2016). CD204 has been shown to be important in M2 polarisation in murine models (Labonte et al. 2017). Consequently, the predominant cells noted in this study of healthy oral mucosa may represent M2-polarised tissue macrophages with dendritic cell morphology.

Iba1 immunoreactivity was the only significantly different variation in cell frequency between most niches, except the alveolar mucosa and palatoglossal folds. Ionised calcium-binding adaptor molecule 1, also known as Iba1, is a microglia or macrophage-specific calcium-binding protein. The observed variation in Iba1 cell density supports that the immune landscape of the oral cavity in dogs is not uniform but rather compartment-specific and suggests a specific distribution of macrophages within the oral cavity. Considering the oral mucosa has different functional types, including lining, masticatory and specialised mucosa, such compartmentalisation may

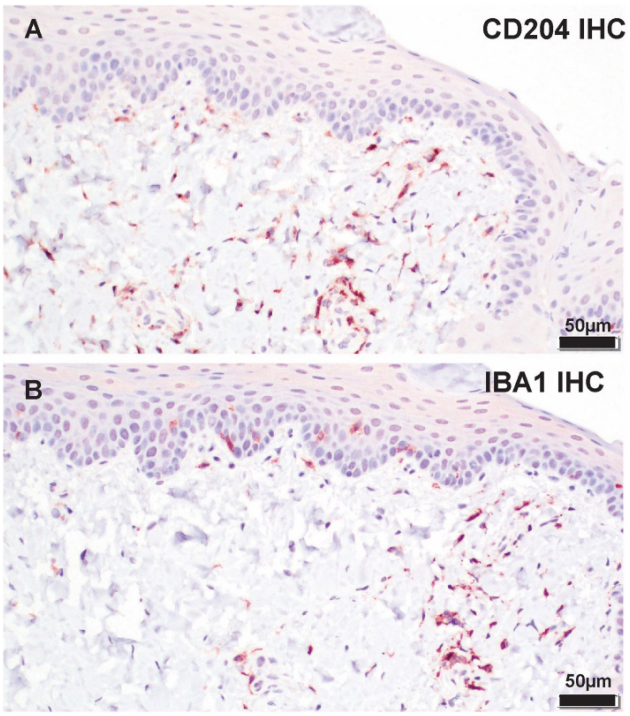

FIGURE 8 | Floor of the mouth (200× magnification; bar = 50 μm). (A) IHC for CD204. (B) IHC for Iba1.

be linked to differences in the histomorphometric qualities of the oral mucosa and the immune surveillance requirements of the various oral regions (Belz and Heath 1995; Winning and Townsend 2000). Another reason for the lack of uniformity may be the microenvironment of each oral compartment (Aframian, Davidowitz, and Benoliel 2006; Ruparell et al. 2020).

The variation between CD204-positive and Iba1-positive macrophages suggests a potential diversity of macrophage subtypes in the submucosa, each with different roles, as has been reported in other species (Gordon, Plüddemann, and Martinez Estrada 2014; Hirayama, Iida, and Nakase 2017; Moutsopoulos and Konkel 2018). Considering that most of the work validating the Iba1 marker has been done in mice, this variation can also be due to differences in the cell type they are labelling (i.e., dendritic cell versus macrophage) or differences in the epitope sensitivity (Matsumoto et al. 2008; Wijesundera et al. 2014; Ibanez et al. 2019). Of no less importance is the fact that 1ba-1-positive cells in our study were most numerous in the floor of the mouth. This discovery suggests that sublingual mucosal vaccines may be effective in canine patients by efficiently triggering immune responses in both the mucosal and systemic compartments through macrophages and dendritic cells (Hovav 2014).

In terms of lymphocyte population, our study did not find significant differences in the densities between niches for this cell type; however, CD3-positive cells were the only lymphocytes found in the epithelium, and the sub-epithelium showed a predominance of CD3 over CD20-positive cells. CD3 (cluster differentiation 3) is a T cell co-receptor involved in activating both cytotoxic as well as helper T cells, both of which are key components of the adaptive immune response in the oral cavity. Their presence in the epithelium indicates a surveillance role at the mucosal surface, possibly responding to potential pathogens or maintaining tissue integrity. CD20, on the other hand, is expressed on the surface of all B cells and progressively increases in concentration until maturity. Though not predominant, the presence of CD20-positive lymphocytes in various oral compartments may indicate a role for B lymphocytes in local immune surveillance and responses in dogs that is different to specific pathogen-free cats in which no B-lymphocytes were found (Arzi et al. 2011). The predominance of CD3-positive cells over CD20-positive cells in the sub-epithelial layer is interesting. The prevalence of T cells in this layer suggests an active immune response involving T cell-mediated mechanisms. This composition could indicate a state of immunological readiness or ongoing immune surveillance in the oral mucosa, which is consistent with studies in cats and humans (Arzi et al. 2011; Moutsopoulos and Konkel 2018).

Immunoreactivity against CD79α, another marker for B cell lineage, was also tested. Expression of CD79 temporally precedes CD20; however, they are expressed at the same time for most of a B cell's life (van Noesel et al. 1991). Immunoreactivity for CD20 was significantly higher than CD79α in the submucosal tissues in this cohort. CD20 is a marker for B cells, and CD79α is a component of the B cell receptor complex. This prevalence of CD20 over CD79α might suggest that these different types of B cells in the submucosal tissues may have different functions. This distinct pattern of immunoreactivity is different in cats, where no B cells were detected in any niche or compartment (Arzi et al. 2011). Environmental exposures and pathogens that dogs and cats encounter can differ and their immune systems can be distinct (Day 2016). Further investigation would be needed to understand the precise nature of the immune response and the factors influencing the distribution of these B cell markers in the submucosal tissues.

When interpreting these results, it is important to consider that this study focused on healthy, client-owned dogs. Variations in immune cell distributions may differ in older dogs, in specific pathogen-free patients, in those with different diets or at different health states including oral disease, and in those who may have systemic disease resulting in immunosuppression or are immunocompromised (Cortese et al. 2015; Pereira et al. 2019). We also acknowledge the challenge posed by our small sample size affecting the generalisability of our findings to broader canine populations. The lack of significance for the other factors could also be attributed to the low sample size. Furthermore, our study did not specifically stain mast cells in the oral compartments, as these were not detected in H&E. This may have underestimated their frequency in the different compartments, as this is largely dependent on the granularity of these cells (Sabattini et al. 2018). This omission is noteworthy, as mast cells play essential roles in wound healing and defence against pathogens (Trabucchi et al. 1988). Lastly, the results may have been influenced by freezing prior to sample acquisition. Though no obvious effect could be detected, this was not statistically evaluated due to the small sample size.

Further studies to expand our understanding of oral immunity in dogs should investigate fresh samples, multiple markers simultaneously, gene expression and perform functional assays to interrogate activity and function of both myeloid and lymphoid

cells in the healthy, diseased and treated oral mucosa of dogs. Additionally, investigation of the oral microbiota composition may reveal a role in shaping the oral immune landscape (Ruparell et al. 2020). Furthermore, the gingival sulcular tissues are a promising avenue of research and were not evaluated in this study (Johannessen et al. 1990; Alcoforado et al. 1990).

In conclusion, this study highlights the compartment-specific differences within the oral cavity of healthy dogs, including the significant difference in Iba1 cell densities, potentially highlighting a significant role of macrophages in oral health. These discoveries enhance our understanding of oral immunity in dogs and provide a foundation for future investigations.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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